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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/62, C07K 19/00, 16/28, 14/435, A61K 39/35, 39/395 — A1

(11) International Publication Number: **WO 97/07218**

(43) International Publication Date: 27 February 1997 (27.02.97)

(21) International Application Number: PCT/EP96/03616

(22) International Filing Date: 16 August 1996 (16.08.96)

(30) Priority Data:
9516760.7 16 August 1995 (16.08.95) GB

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ALLERGEN-XCD32 FUSION PROTEINS

(57) Abstract

Fusion proteins comprising one or more antigens and one or more moieties interacting with human Fcγ receptor II (FcγRII) (CD32).

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ALLERGEN -XCD32 FUSION PROTEINS

The present invention relates to complexes of human IgG and antigen/allergen (or a combination of antigens/allergens). It concerns fusion proteins between anti-CD32 molecules and antigen/allergen (or a combination of antigens/allergens). Allergens are defined herein as antigens to which atopic patients respond with allergic reactions. Antigens as used herein can be of various origins, e.g. environmental allergens (e.g. house dust mite, birch pollen, grass pollen, cat antigens, cockroach antigens), or food allergens (e.g. cow milk, peanut, shrimp, soya), or a combination of both, or non-relevant antigens such as bovine serum albumin (BSA).

Allergy is a disease in which IgE antibodies mediate activation of effector cells (mast cells, basophils, eosinophils) but also enhance antigen presentation through low and high affinity receptors for IgE (B cells, monocytes, dendritic cells). These actions of IgE can be countered by specific IgG antibodies which interact with CD32 (FcγRII) on both effector and inducer cells. Therefore, allergy should be regarded as a disease with a disbalance of Th1 versus Th2 cells. The success of the IgG antibodies in counteracting IgE in vivo depends on the relative concentration of specific IgE over specific IgG, and also on the isotype of IgG. All human isotypes have low affinity for CD32, which can be overcome by complex formation with allergen, but many allergens do not form large enough complexes with IgG molecules to allow stable binding to CD32.

To date two forms of active vaccination using allergens are used. The most common is the so-called "immunotherapy", which depends on frequent immunizations with relatively high concentrations of allergens. This technique is only moderately effective in a minority of allergic diseases such as bee sting allergy and in some cases of rhinitis and conjunctivitis, and recently some reports have shown effectiveness in asthma and atopic dermatitis. Classically the subcutaneous route is used for administration of the allergens, but recently this route has been compared to oral application or even local application, the results are generally positive but not always consistent. A more recent approach for immunotherapy, the so-called Saint-Remy technique (see e.g. EP 0 178 085 and 0 287 361), uses autologous IgG antibodies complexed in vitro to the relevant allergens. This approach allows far smaller amounts of allergen to be applied with less side effects.

The mechanism behind both therapies is unclear. In the classical approach there seems to be a beneficial effect if the therapy induces an increase in specific IgG antibodies, although not every significant increase of specific IgG is correlated with successful immunotherapy. A possible reason for this is the relatively low affinity of IgG antibodies for CD32 on B cells, monocytes and mast cells. The Saint-Remy approach selects the specific IgG antibodies from the patient, which are subsequently mixed with relevant allergens in vitro. This way they assure that the allergen cannot react freely with cells or other antibody isotypes on cells, such as IgE on mast cells. In addition anti-idiotypic antibodies are presumably raised against the specific IgG molecules, and subsequently will prevent allergy, although this has not been confirmed by experimental data.

IgG molecules are important in directing the immune response away from B cells to monocytes. The Saint-Remy approach should therefore work when general allergen-specific IgG is used instead of donor-specific allergen-specific IgG. However, a problem with the Saint-Remy approach lies in the low affinity of human IgG antibodies for their Fcγ receptors on B cells, in contrast to IgE which binds readily to almost all antigen-presenting cells, including B cells, leading to spreading of allergy and induction of Th2 cells. The use of complexes should by-pass the low affinity problem, due to enhanced avidity of the complex as compared to single IgG molecules; however, again, the combination of allergen/IgG in complexes is unfavourable, because the natural number of epitopes of allergens for binding human IgG molecules is less than five, too few to enhance the avidity of the complex for the receptors.

With the present invention, the risk factors of classical immunotherapy are reduced and the problems encountered in isolating specific IgG molecules and the low affinity of these IgG antibodies for CD32 are circumvented.

IgE bound to mast cells and basophils induces histamine release when cross-linked by antigen. In addition, IgE is able to enhance antigen presentation by human B cells (Figure 1). Otherwise, in the absence of IgE B cells are poor antigen-presenting cells, except for a very small portion of B cells, which have a specific antigen receptor. Antigen uptake can occur through pinocytosis but this takes high antigen concentrations and/or long incubation times.

In Figure 2 it is shown that pinocytosis of allergen over 5 days is able to mimic IgE-mediated antigen presentation, but that IgG-mediated antigen presentation does not lead to stimulation of the T cells. In fact, the data indicates prevention of allergen presentation by complexing allergen to IgG.

In another series of experiments the influence of these IgG-allergen complexes on IgE-mediated antigen presentation or on allergen uptake through pinocytosis was studied. In Figure 3 it is shown that IgG complexes inhibit in a dose-dependent manner IgE-mediated antigen presentation, further, antigen presentation after pinocytosis is inhibited. This effect is not antigen-specific (IgG/BSA complexes inhibit Der P1 - specific stimulation), monomeric (non-complexed) IgG does not inhibit antigen presentation.

Toxicity of the IgG/allergen complexes is excluded by the experiment shown in Figure 4. IgG3-Der P1 complexes (pre-)incubated with human monocytes are able to induce a normal T cell response. This implies that IgG/allergen complexes inhibit allergen presentation by B cells and at the same time stimulate monocytes and dendritic cells. Similarly, aCD32 antibodies or aggregated IgG coated to the bottom of the wells in which the stimulations are performed, also inhibit antigen presentation by B cells (Figure 5). The efficiency with which CD32 targeted molecules inhibit antigen presentation is most likely dependent on the amount of cross-linking of the CD32 molecule. However, targeting antigen to CD32 on B cells, as in natural IgG/antigen complexes (Figure 2) or as in the aCD32/antigen fusion proteins according to this invention (Figures 13a and 13b), does not lead to antigen presentation of the antigen in the complex. On the other hand the same complexes targeted to monocytes do induce an antigen-specific T cell response (Figures 4, 13a and 13b). This phenomenon can be explained by the presence of CD32B on B cells, whereas monocytes and also dendritic cells express CD32A. Therefore IgG/antigen complexes as well as aCD32/antigen fusion proteins direct the immune response to cells of the monocytic and dendritic cell lineage, which induce T cell responses of mainly Th1 type B cells, and other APC which predominantly express CD32B will not present the antigen in the complex, therefore these complexes prevent (further) induction of Th2 cell responses by the B cells. The interaction between the complex and CD32 does not depend on heavy cross-linking (more than 2 CD32 molecules), provided the binding of the complex is strong enough to allow stable interaction. In fact it has been shown that binding of very large complexes, which induce multiple crosslinking of CD32 (mimicked by coated

aggregated IgG) on monocytic cells leads to downregulation of co-receptors, such as CD80, important for antigen-specific stimulation of T cells (Table 1).

Apart from antigen presentation, IgG/antigen complexes or aCD32/antigen fusion proteins according to the invention also inhibit Ig production by normal human B cells. In Figure 14 it is shown that tonsilar B cells stimulated with aCD40 and IL-4 in the presence of aCD32 antibodies or aggregated coated human IgG, which efficiently interact with CD32, are no longer able to produce antibodies. Treatment with aCD32 F(ab) fragments is as efficient as the use of complete antibodies (Figure 15), indicating that for inhibition of Ig production after stimulation of aCD40 and IL-4 no cross-linking of the receptor is necessary. B cells which are stimulated by antigen through their B cell receptor (BCR) need co-crosslinking of BCR and CD32. The downregulation of Ig production is reversible and time dependent. When the aCD32 antibodies are added to the B cells 4 days after aCD40 plus IL-4 stimulation, no inhibition of Ig production is seen. In Figure 16 it is shown that also antigen-specific induction of antibody production is inhibited by aCD32 treatment of B cells.

In nature, IgG molecules control the response of B cells through interaction with CD32. Especially the effects of IgE antibodies, which in general have a positive effect on (CD23 positive) B cells, can be counteracted by IgG molecules. This prevents the organism from hyperreactive immune reactions to antigens. In allergy, where IgE antibodies are abundantly present, clearly the natural IgG antibodies have failed to control the immune response. Interestingly, in man IgE and IgG4 are regulated by the same interleukin, namely IL-4. However, IgG4 has the lowest affinity of all human IgG subclasses for CD32.

The present invention concerns fusion proteins comprising

a) one or more antigens and

b) one or more moieties, such as from antibody molecules, interacting with human Fcγ receptor II (FcγRII) (CD32),

hereinafter briefly named "the fusion proteins according to the invention".

The fusion proteins according to the invention overcome the problems due to the low affinity of human IgG molecules to CD32. By combining an aCD32 antibody having a $K_d < 10^{-6}$ with antigen, both negative (B cells) and positive effects of natural IgG molecules

are obtained, including selective stimulation of the immune system leading to Th1/Th0 memory induction in the absence of antibody production. The effect is harmless and directs the immune response to antigen-presenting cells which express CD32, whereby the cells which predominantly express CD32A mediate antigen presentation leading to induction and activation of Th1 cells as a result of the IL-12 produced by the CD32A-expressing antigen-presenting cells.

The fusion proteins according to the invention preferably are devoid of heavy cross-linking. The number of binding sites for CD32 preferably is limited so as to avoid down-regulation of stimulatory co-receptors such as CD40, CD80 or CD86.

They also knock out the effector function of mast cells which carry IgE-specific parts of the fusion protein. They have the following unique characteristics:

1) silencing of B cell-mediated antigen presentation (arresting induction of Th2 cells);
2a) arresting IgE switch induction;
2b) arresting Ig production in B cells;
3) stimulating the T cell compartment through interaction with monocytic cells and/or dentritic cells (stimulation of Th1 cells); and
4) silencing of mast cells carrying IgE specific for parts of the fusion protein.

They comprise a) one or more antigens and b) one or more moieties, such as from antibodies, interacting with human Fcγ receptor II.

The parts of the fusion protein which interact with the Fcγ receptors II may e.g. be either

1) complete or incomplete (modified) human or humanized IgG antibody moieties, as long as interaction with these receptors is still possible, which implies that the whole or part of the Fc fragment should be present; or
2) human or humanized aCD32 antibody moieties, or parts thereof, e.g. Fab fragments, which still specifically recognize and bind to FcγRII (CD32) antigen such as expressed on B cells, mast cells, monocytes and dentritic cells,

e.g. manipulated human or humanized aCD32 or IgG antibody moieties, or parts thereof, which recognize FcγRII (CD32) with higher affinity than the native aCD32 or IgG antibodies.

The antigens may be from complete proteins or parts thereof still having epitopes for T cells on the sequences present in the fusion protein. Any antigen to which allergic patients respond with IgE-mediated hypersensitivity reactions can be used, such as allergens in atopic dermatitis, allergic asthma, allergic rhinitis and allergic conjunctivitis. The most common environmental allergens are: house dust mite, birch pollen, grass pollen, cat, cockroach. Each of these allergens has one or more "major allergens" (e.g. for house dust mite the major allergen is Der P1; for birch pollen the major allergen is Bet V1). However, complete antigens are not necessary, because the fusion protein will normally only induce T cell responses, and T cells respond to small (8-12 amino acids long) peptides. Therefore a selection of T cell epitopes can be included in the fusion protein for each allergen, thus reducing the size and molecular weight of the complex. Thus the fusion proteins may be produced from one or more T cell epitope - containing DNA stretches, rather than from the genes for the complete antigens. Overlapping cross-reactive epitopes between allergens are preferred. The fusion protein should specifically bind to CD32 and contain one or more, e.g. two or more T cell epitopes for one or more antigens/allergens. To allow for correct antigen processing, DNA stretches slightly longer than the actual T cell epitope should preferably be included in the preparation of the fusion proteins.

For fusion to gene coding for the aCD32 antibody, preferably short DNA sequences derived from cloned genes of major allergens are used, such as house dust mite major allergen I (Der P1) or birch pollen allergen (Bet V1). These short DNA sequences contain the genetic code for one or more T cell epitopes which after processing appear on the surface of antigen-presenting cells and therefore induce an immune response in the responding allergen-specific T cells. For Der P1 the majority of the T cell epitopes can be found in the sequence at positions 101-143 in amino acid one-letter code (SEQ. ID. No.1):

```
  QSCRRPNAQRFGISNYCQIYPPNANKIREALAQPQRYCRHYWT
101       110       120       130       140
```

Especially, the amino acid sequence at positions 101-131 in amino acid one-letter code (SEQ. ID. No.2):

```
  QSCRRPNAQRFGISNYCQIYPPNANKIREAL
101       110       120       130
```

contains at least three T cell epitopes, which bind to a number of HLA class II molecules.

The fusion between e.g. aCD32 and antigen(s) can be effected either at the protein level (chemical fusion) or at the gene level (recombinant fusion protein), and the invention also comprises a process for producing fusion proteins as defined above. It is carried out in conventional manner, and preferably comprises the use of recombinant gene techniques or chemical cross-linking.

Recombinant fusion proteins are preferred.

Preparation using recombinant gene techniques may be carried out by known methods, e.g. in the following way: a gene segment containing the antigen-binding site and parts of the Fc region down to the CH2 exon of an aCD32 monoclonal antibody (e.g. clone IV.3) is PCR-amplified from e.g. IV.3 cDNA and cloned. Appropriate RNA is purified as a source for PCR-amplification, e.g. from house dust mites for amplification of the Der P1 gene. The allergen gene is co-ligated with the isolated heavy chain segment into an appropriate mammalian expression vector such as p350. In addition, the complete corresponding light chain gene of e.g. IV.3 is isolated and cloned into an appropriate mammalian expression vector such as p345, which has features similar to p350. Both recombinant plasmids are used for co-transfection in e.g. COS cells to allow release of the resultant recombinant fusion protein into the culture medium. Purification of the product is done preferably by immunoaffinity purification, based on anti-light chain antibody columns, which are readily available in large quantities.

Alternatively, a single chain antibody specific for CD32 (scFv) derived from an appropriate phage display library, e.g. as described by De Kruif et al., Proc. Natl. Acad. Sci. USA 92 (1995) 3938-3942 can be used to make a recombinant fusion protein with the genes of cloned allergens (Figure 6). DNA is extracted from binding phages and a DNA fragment containing a semi-synthetic VHDJH region fused to one of the VL chains via a flexible linker (scFv fragment) is isolated and cloned. Fusions with e.g. the Der P1 gene are accomplished using defined synthetic oligonucleotides coding for T-cell epitopes from Der P1 and other major allergens selected by their high (T cell) crossreactive potential in order to cover as many patients as possible with one fusion protein. These oligonucleotides are co-ligated with the scFv antibody fragment into an appropriate mammalian expression vector. The recombinant vector, e.g. a

plasmid is stably transfected into appropriate cells such as COS and/or CHO cells and the resultant fusion protein is purified from the cell supernatant, e.g. by immunoaffinity chromatography as described above.

The resultant gene construct may be expressed e.g. in CHO cells, especially for large scale production; however, other systems for production by recombinant gene techniques are also appropriate, especially when only T cell epitopes are used, i.e. there is no need for glycosylation. The aCD32 antibodies can be obtained e.g. from a human Ig phage display library which contains only F(ab) fragments of the natural antibody. However, the source of the genetic material which codes for either antibody or antigen/allergen is not critical.

Preparation by chemical cross-linking may also be carried out by known methods, e.g., for a fusion protein between Mc.a-CD32 and Der P1, by using the methods described by Calsson et al. [Biochem.J. 173 (1978) 723-737], Cumber et al. [Meth.Enzymol. 112 (1985) 207-224] and Peeters et al. [J.Immunol.Meth. 120 (1989) 133-143].

Briefly, Der P1 and Mc.a-CD32 are separately derivatized with SPDP at molar ratios of 1/20 and 1/5 respectively for introducing 2-pyridyldisulphide residues. After mild reduction and desalting by gel chromatography, the SPDP-derivatized Mc.a-CD32 (whose disulphide groups are reduced to thiol groups) are incubated with the SPDP-derivatized Der P1 at a molar ratio of 1/1.6. The resulting Der P1-Mc.a-CD32 product is then purified, for example by gel filtration on e.g. Superose-12 and by anion exchange chromatography on e.g. FPLC Mono-Q.

The starting materials are either known or may be prepared according to known procedures or analogously to known procedures or analogously as described herein, e.g. in the Example.

The fusion proteins according to the invention are useful for the prevention and/or treatment of allergies, particularly of food allergies. It is not uncommon for patients who suffer from anaphylactic response to a particular allergen, also to suffer from such a response to one or more other allergens. It is possible by the method of the present invention to desensitize such a patient in respect of two or more allergens simultaneously by administering a fusion protein including antigens against each of these allergens. Preferably the fusion proteins according to the invention will be used for the prevention and/or treatment of allergy in newborn children at risk of food allergy e.g. to milk, or of established allergies in patients with allergy against the allergen(s) which is (are) included in the particular fusion protein used.

For these indications the appropriate dosages will, of course, vary depending upon, for example, the particular fusion protein used, the host, the mode of application and the intended indication. However, in general, satisfactory results are indicated to be obtained with one to three vaccinations over 1-2 years, but if necessary repeated additional vaccinations can be done. It is indicated that for these treatments the fusion proteins of the invention may be administered in dosages and with an application schedule similar as conventionally employed.

The invention therefore also concerns the use of a fusion protein as defined above in the prevention and/or treatment of allergies, including food allergies, and a method of treating allergies which comprises administering to a subject in need of such treatment a prophylactically or therapeutically effective amount of a fusion protein as defined above together with at least one conventional pharmaceutically acceptable carrier or diluent, as well as fusion proteins as defined above for use as a pharmaceutical, especially as an anti-allergic agent.

The fusion proteins according to the invention may be admixed with conventional pharmaceutically acceptable diluents and carriers and, optionally, other excipients and administered parenterally, intravenously or enterally, e.g. intramuscularly, or subcutaneously. The concentrations of the fusion protein will, of course, vary depending i.a. on the compound employed, the treatment desired and the nature of the pharmaceutical form.

The invention thus also includes pharmaceutical compositions comprising a fusion protein as defined above together with at least one pharmaceutically acceptable carrier or diluent.

It further concerns a process for the preparation of a medicament against allergies which comprises mixing a fusion protein as defined above together with a pharmaceutically acceptable carrier or diluent, and the use of a fusion protein as defined above for the manufacture of a medicament for the prevention and/or treatment of allergies, including food allergies.

The following abbreviations are used herein:

aCD32	antibody to CD32 (anti-CD32 antibody)
Ag	antigen
APC	antigen-presenting cell
BCR	B cell receptor
Bet V1	major allergen of birch pollen
BSA	bovine serum albumin
CNBR	cyanobromide
Der P1	major allergen of house dust mite (Dermatophagoides pteronyssinus)
DPT	antigen of house dust mite
DTT	dithiothreitol
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorter
FcγRII	human Fcγ receptor II (= CD32)
Fig.	Figure number
FPLC	fast pressure liquid chromatography
GaM	goat anti-mouse antibody
HAc	acetic acid
HLA	human leukocyte antigen
HPHT	hydroxyapatite
IC	immune complex
Ig	immunoglobulin
IL-12	interleukin-12
LST	lymphocyte stimulation test
min	minutes
MR	molar ratio
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
p-NPP	p-nitrophenylphosphate
sd	standard deviation
SPDP	N-succinimidyl 3-(2-pyridylthio)propionate

In the following Example, which illustrates the invention without limiting it, all temperatures are in degrees Celsius.

Example: Preparation and purification of Der P1--monoclonal anti-CD32 fusion protein

A) **Method:**

Der P1 is covalently conjugated to Mc.a-CD32 by using SPDP, a bifunctional coupling reagent. In brief, Der P1 and Mc.a-CD32 are separately derivatized with SPDP at molar ratios of 1/20 and 1/5 respectively for introducing 2-pyridyldisulphide residues. After mild reduction and desalting by gel chromatography, the SPDP-derivatized Mc.a-CD32 (whose disulphide groups are reduced to thiol groups) are incubated with the SPDP-derivatized Der P1 at a molar ratio of 1/1.6. The resulting Der P1–Mc.a-CD32 conjugate is purified by gel filtration on Superose-12 and by anion exchange chromatography on FPLC Mono-Q.

B) **Materials:**

a) SPDP: molecular weight 312.4; 25.61 mM stock solution (8 mg/ml) in dimethylformamide, prepared immediately before coupling.

b) Der P1: purified by immunoaffinity chromatography on Mc.a-Der P1(4C1/B8/3F8) covalently coupled to CNBR-Sepharose 4B. Molecular weight 28 kD; isoelectric point 5.0 (PHAST-IEF); sterile filtered, diluted in PBS.

c) Monoclonal anti-CD32(IV.3): isotype: mouse IgG2b-κ chain; molecular weight 150 kD; isoelectric point 6.2 (PHAST-IEF); purified from culture medium supernatant on Protein-A and HPHT and dialysed against PBS.

Reaction mixture A:

To 1.2 ml of Der P1 solution (0.84 mg) is added 23.4 µl of SPDP stock solution (20 molar excess) and the mixture is stirred in a 2.5 ml conical reactive vial (Pierce) for 45 minutes at room temperature (22°). The reaction mixture is filled up to 2.5 ml with PBS and unconjugated SPDP and released N-hydroxysuccinimide is removed by desalting on a 9.1 ml Pharmacia PD-10 disposable desalting column (Sephadex G-25) equilibrated with 50 ml of PBS. After application the fraction containing the 2-pyridyldisulphide-activated Der P1 is eluted and pooled with 3.5 ml of PBS. The concentration is about 0.17 mg/ml, corresponding to 0.6 mg.

Estimation of the degree of substitution with 2-pyridyldisulphide:

A 100 µl sample is filled up to 150 µl with PBS and 50 µl of 150 mM DTT diluted in PBS is added. The concentration of pyridine-2-thione released after DTT addition can be determined by measuring the absorbence at 343 nm (molar extinction coefficient: 8080 $M^{-1}cm^{-1}$) and corresponds to the introduced 2-pyridyl-disulphide residues. For a measured OD 343 nm increase of 0.074 (corrected for dilution) the substitution degree is calculated as 1.5 Mol 2-pyridyldisulphide / Mol Der P1.

Reaction mixture B:

To 0.5 ml of Mc.a-CD32 solution (4.2 mg) is added 5.5 µl of SPDP stock solution (5 molar excess) and the mixture is stirred for 45 min at room temperature. The reaction mixture is applied on a 2.5 ml Pierce GF-5 disposable desalting column (equilibrated with 10 ml of 0.1M Na-acetate/HAc buffer, 0.1M NaCl, pH 4.5). After application, the desalted fraction is eluted and pooled with 1.5 ml of acetate buffer (pH 4.5) and filtered with a 0.22 µm MILLEX-GV (low protein binding) filtration unit.

Introduction of SH-groups:

Protein-bound 2-pyridyldisulphide groups are converted to protein-bound thiol groups by reducing with DTT. 1.5 ml of desalted sample is incubated under stirring with 1 ml of 62.5mM DTT (diluted in acetate buffer) at room temperature for 30 minutes (final DTT concentration 25mM). Because 2-pyridyl disulphide increase their electrophilicity at acidic pH the reduction can still be carried out at low pH, where native protein disulphide bounds will not be affected.

For the measured OD 343 nm increase of 0.447, the substitution degree is calculated as 7 Mol thiol groups / Mol Mc.a-CD32.

The reaction mixture is applied on a Pharmacia PD-10 column (equilibrated with 50 ml of PBS) and after application, the desalted fraction is eluted and pooled with 3.5 ml of PBS. The sample is concentrated to 0.6 ml with a CENTRIPLUS-10 (YM10 membrane) concentration unit. The concentration is about 3.1 mg/ml, corresponding to 1.86 mg.

C) **Coupling procedure:**

3.3 ml (0.56 mg) of Der P1 (pyridine-disulphide activated) and 0.6 ml (1.86 mg) of Mc.a-CD32 (SH-activated) are mixed and stirred in a 5 ml Pierce reactive vial for 2 hours at room temperature (22°) and 15 hours at +4°. The molecular ratio of Der P1 / Mc.a-CD32 in the reaction mixture is 1.6/1. The coupling reaction is monitored by measuring the increase of OD 343 nm due the increase of released pyridine-2-thione:

Time	OD (343 nm)
Start	0.1112
15min	0.1257
30min	0.1342
45min	0.1412
60min	0.1470
75min	0.1519
120min	0.1590

D) **Purification of conjugate:**

Superose-12 :

The reaction mixture (3.9 ml) is filtered with a 0.22 µm MILLEX-GV filtration unit and concentrated to 0.5 ml with a Centriplus-10 concentration unit. The final concentration of the conjugate is 3.7 mg/ml, corresponding to 1.9 mg.

The sample is applied on a Superose-12 (HR10/30) column (Pharmacia) equlibrated with PBS. Bed volume 24 ml; flow rate 0.2 ml/min; volume/fraction 0.4 ml; recorder chart speed 1.5 mm/min; pressure 0.3 MPa; scanning wavelength 280 nm. The eluted high molecular weight fractions are divided according to the elution profile in 3 pools:

1.2 ml Pool A: fraction# 21-23
1.2 ml Pool B: fraction# 24-26
1.2 ml Pool C: fraction# 29-31

The pools are sterile filtered (0.22 µm MILLEX-GV) and stored under sterile conditions at +4°. The total protein concentration is about: Pool A: 100 µg/ml; Pool B: 200 µg/ml; Pool C: 390 µg/ml.

FPLC-Mono Q :

1 ml of Pool C (390 µg) is dialysed against 20 mM of ethanolamine/HCl, 0.01 % NaN_3 buffer pH 9.0 and purified by ion-exchance chromatography on FPLC-Mono Q (HR5/5) anion exchanger. Starting buffer A: 20 mM ethanolamine/HCl, 0.01%NaN_3 pH 9.0; limiting buffer B: A + 0.5 M NaCl pH 9.0; flow rate 1 ml/min, volume/fraction 1 ml; recorder chart speed 5 mm/min; pressure 1.2 Mpa; scanning wavelenght 280 nm. After application, a linear gradient program is started for 30 minutes (conductivity 50 - 1260 µS/cm). The relevant peak is eluted in fraction #8 (at 340 µS/cm). The protein concentration is about 45 µg/ml (OD 280 nm, $E_{1\%}$ 14.0). The sample is dialysed against PBS and sterile filtered. The protein concentration of the final purified material is 20 µg/ml (OD 280 nm).

E) Analytical determinations:

a) Total protein concentration:

Total protein concentrations are estimated according to Bradford, using BIO-RAD Protein Assay Kit I; standard: bovine IgG.

b) Determination of Der P1, of Mc.a-CD32 and of Der P1--Mc.a-CD32 conjugate by ELISA:

Solid phase ELISAs are performed in PVC microtiter plates. The coating buffer 0.1 M $NaHCO_3/Na_2CO_3$, 0.01% NaN_3 pH 9.6, PBS with 0.05% Tween-20 is used as washing solution and 2 % fetal calf serum in washing buffer as diluent for samples, biotin- and enzyme conjugates. The substrate is 1 mg/ml p-NPP diluted in 1 M diethanolamine/HCl buffer pH 9.8. The stopping solution is 2 M NaOH. All incubation steps are done in a humidified chamber. Equipment for sample processing and OD reading at 405 nm is Beckman Biomek-1000 laboratory workstation. Quantitative evaluation: Beckman Immunofit; curve fitting: 4-parameter logic.

b)1) Determination of Der P1 (Fig. 7):

Coating: 100 µl/well Mc.a-Der P1(5H8) 10 µg/ml overnight at +4°. After washing, 100 µl/well sample is added:

a) Der P1 (starting material for coupling) (250-0.49 ng/ml) as standard
b) Superose-12 Pool A (4000-7.81 ng/ml)
c) Superose-12 Pool B (4000-7.81 ng/ml)
d) Superose-12 Pool C (4000-7.81 ng/ml)

Incubation for 2 hours at +37°. After washing, 100 µl/well Mc.a-Der P1(4C1/B8/3F8)-Biotin conjugate diluted 1/500 is added and incubated for 2 hours at +37°. After washing, 50 µl/well streptavidin-alkaline phosphatase conjugate diluted 1/1000 is added and incubated for 1 hour at +37°. After washing, 100 µl/well substrate is added, incubated for 60 minutes at 37° and stopped. Quantitative evaluation from standard curve:

Superose-12 Pool A: 16.1 µg/ml
Superose-12 Pool B: 54.9 µg/ml
Superose-12 Pool C: 135 µg/ml

b)2) Determination of Mc.a-CD32 (mouse IgG2b) (Fig. 8):

Coating: 100 µl/well goat Pc.a-Mouse IgG2b (Southern) 5 µg/ml overnight at +4°. After washing, 100 µl/well sample is added:

a) Mc.a-CD32 (starting material for coupling) (1000-1.96 ng/ml) as standard
b) Superose-12 Pool A (8000-15.63 ng/ml)
c) Superose-12 Pool B (8000-15.63 ng/ml)
d) Superose-12 Pool C (8000-15.63 ng/ml)

Incubation for 2 hours at +37°. After washing, 50 µl/well goat Pc.a-Mouse IgG2b-alkaline phosphatase conjugate (Southern) diluted 1/1000 is added and incubated for 2 hours at +37°. After washing, 100 µl/well substrate is added, incubated for 15 minutes at room temperature and stopped. Quantitative evaluation from standard curve:

Superose-12 Pool A: 17.7 µg/ml
Superose-12 Pool B: 58.8 µg/ml
Superose-12 Pool C: 190 µg/ml

Evaluation of molar ratio Der P1 / Mc.a-CD32 in conjugate accord ELISA:

Superose-12 Pool A: 4.9 / 1
Superose-12 Pool B: 5.0 / 1
Superose-12 Pool C: 3.8 / 1

b)3) Detection of Der P1--Mc.a-CD32 conjugate:

b)3)1) Coating: Mc.a-Der P1 (Mouse IgG2a) (Fig. 9):

Coating: 100 µl/well Mc.a-Der P1(5H8) 10 µg/ml overnight at +4°. After washing, 100 µl/well sample is added:

a) Der P1 (starting material for coupling) (4000-7.81 ng/ml)
b) Superose-12 Pool A (4000-7.81 ng/ml)
c) Superose-12 Pool B (4000-7.81 ng/ml)
d) Superose-12 Pool C (4000-7.81 ng/ml)

Incubation for 2 hours at +37°. After washing, 50 µl/well goat Pc.a-Mouse IgG2b-alkaline phosphatase conjugate (Southern) diluted 1/1000 is added and incubated for 2 hours at +37°. After washing, 100 µl/ well substrate is added, incubated for 30 min at room temperature and stopped. Mc.a-Der P1 - bound Der P1--Mc.a-CD32 conjugate is detected by the Mc.a-CD32 partition in each Superose-12 purified fraction pool.

b)3)2) Coating: Pc.a-Mouse IgG2b (Fig. 10):

Coating: 100 µl/well goat Pc.a-Mouse IgG2b (Southern) 5 µg/ml overnight at +4°. After washing, 100 µl/well sample is added:

a) Der P1 (starting material for coupling) (1000-1.95 ng/ml)
b) Superose-12 Pool A (8000-15.63 ng/ml)
c) Superose-12 Pool B (8000-15.63 ng/ml)
d) Superose-12 Pool C (8000-15.63 ng/ml)

Incubation for 2 hours at +37°. After washing, 100 µl/well Mc.a-Der P1(4C1/B8/3F8)-Biotin conjugate (mouse IgG1) diluted 1/500 is added and incubated for 2 hours at +37°. After washing, 50 µl/well streptavidin-alkaline phosphatase conjugate diluted 1/1000 is added and incubated for 1 hour at +37°. After washing, 100 µl/well substrate is added, incubated for 60 min at 37° and stopped. Pc.a-Mouse IgG2b - bound Der P1--Mc.a-CD32 conjugate is detected by the Der P1 partition in each Superose-12 purified fraction pool.

c) **Determination of the molecular weight of Der P1--Mc.a-CD32 conjugate by native polyacrylamide gel gradient electrophoresis:**

The molecular weight of the Der P1--Mc.a-CD32 conjugates in the Superose-12 purified fraction pools is estimated with a native PHAST gel 4-15% gradient (Pharmacia) (separation range: 1000 kD - 150 kD) compared to native high molecular weight standard proteins (kit, Pharmacia).

Detection: silver staining (Silver staining kit, Pharmacia) (Fig. 11)

Evaluation:

Superose-12 Pool A: band at MW 700 kD
Superose-12 Pool B: band at MW 460 kD, 330 kD
Superose-12 Pool C: band at MW 330 kD, 170 kD

The molecular weight of the final FPLC-Mono Q purified Der P1--Mc.a-CD32 conjugate is estimated as 330 kD (Fig. 12).

F) **Test results:**

a) **Antigen-specific stimulation of T cell clone CFTS4:3.1 with aCD32 Der P1 fusion protein:**

The above chemically linked aCD32 (Medarex clone IV.3) with purified Der P1 is used to stimulate Der P1 - specific T cell clone CFTS4:3.1 in different concentrations using a standard LST. In a first set of experiments pool A and pool B from the chemically fused preparation are used, pool A containing a single band of 700 kD (2 aCD32 molecules fused with 10 Der P1 molecules), and pool B consisting of two bands, at 460 kD and 330 kD (1 aCD32 with 10 Der P1, and 1 aCD32 with 5 Der P1 molecules, respectively).

In a second set of experiments the purified protein from pool C is used, consisting of a single band of approximately 330 kD (1 aCD32 molecule fused with 5 molecules of Der P1). Monocytes and B cells are preincubated for 1 hour at room temperature with the various fractions as indicated in Figures 13a and 13b. As control stimulation 100 µg/ml of DPT is added to T cells plus antigen presenting cells during the complete culture period. B cells are able to stimulate CFTS4:3.1 with DPT but not with the pool A, pool B, or pool C, whereas monocytes are able to

stimulate CFTS4:3.1 with DPT and with pool A, pool B, and pool C. This confirms the previous findings with natural IgG allergen complexes referred to above. In addition this indicates that for antigen stimulation by monocytes CD32 crosslinking is not necessarry, since all fractions are stimulating the T cells equally well.

b) **Inhibition of IgE synthesis:**

Purified human tonsillar B cells are stimulated with aCD40 and IL-4 in the presence and absence of commercially available aCD32 antibodies, added in the indicated concentrations (Fig. 14). After 9 days the supernatants of the cultures are tested for IgE and IgG1 content. All aCD32 antibodies inhibit IgE as well as IgG1 production in a dose-dependent way. In Fig. 15 it is shown that even Fab fragments from the Medarex clone IV.3 are able to inhibit IgE synthesis. This indicates that B cells which are not activated via their BCR can be blocked in their antibody production by monomeric interaction with CD32. For B cells which are stimulated in an antigen-specific way, co-cross-linking between CD32 and BCR is necessary. This implies that e.g. in allergy where B cells take up antigen (e.g. Der P1) through IgE and CD23 and subsequently become stimulated Der P1 - specific Th2 cells leading to IgE production by the B cells, these B cells can be shut down by interaction with CD32 on the B cell surface. Indeed, even in cognate B cell T cell interaction, aCD32 antibodies block antibody synthesis (Fig. 16).

Table 1

Stimulation	% CD80	% CD40	% HLA-DR
day 0 (freshly isolated)	1	26	99
day 1 IFN-γ	31	82	99
day 1 IFN-γ + aggregated IgG	13	61	99
day 1 IFN-γ + aggregated IgG + aCD32	32	82	99

Monocytes (91% CD14 positive) derived from a normal donor by elutriation are incubated with 100 U/ml IFN-γ for 24 hours in the presence or absence of 5 µg/ml aggregated human IgG (coated to the wells, overnight at 4^0) and/or 10 µg/ml monoclonal mouse anti-human aCD32 (Medarex IV.3), and subsequently stained for the indicated markers for FACS analysis. The IFN-γ - induced up-regulation of CD80 and CD40 can be inhibited by heavy cross-linking of Fcγ receptors by aggregated human IgG on the monocytes. Preincubation with aCD32 counteracts the inhibition by aggregated human IgG, indicating that

1) the inhibition by the aggregated human IgG is mediated through CD32, and
2) mere binding to CD32 does not cause the down-regulation of the co-receptors.

HLA-DR expression is not influenced by the treatments.

Explanation of the Figures:

Figure 1: Binding of Der-P1-(3)NIP complexes to CD23 in relation to antigen presentation:

Panel A = fluorescence; Panel B = proliferation.

EBV B cells were pulsed with pre-formed IC's, consisting of a constant concentration of IgE (▲ 7.5 µg/ml, ● 5.0 µg/ml, ■ 2,5 µg/ml) and variable concentrations of Der-P1-(3)NIP.

In panel A binding of the pre-formed IC's to the B cells is shown. IC's with an identical MR are connected with a line and the actual MR (0.1, 0.25, 0.5, 1, 7) is indicated in arabic numbers next to the line. IgE binding reached a plateau at Der P1 concentrations of around 0.2 µg/ml. IgE binding in the absence is Der P1-(3)NIP is shown in the dotted box on the lower part of the right Y-axis.

In panel B antigen presentation by irradiated EBV-B cells pulsed with the same IC's is shown. Here, the connecting lines indicate complexes made with identical IgE concentrations, to emphasize that antigen presentation was not influenced by the MR of the IC's (correlation coefficient = 0.96). Also monomeric complexes [highest Der P1-(3)NIP concentration of each line] were efficiently presented to the T cells. No stimulation of T cells was seen with any of the used Der P1-(3)NIP concentrations (hatched area) in the absence of IgE molecules. Data shows ^{3}H-thymidine incorporation on day 5 (mean ± sd of triplicate wells).

Figure 2: Comparison of antigen presentation with **non-pulsed** B cells:

Free Der P1-(2)NIP left in the culture medium during the whole stimulation period induced a dose-dependent stimulation of the T cells. Autologous (irradiated) EBV-B cells were used as antigen-presenting cells. IgE present in the complexes did not significantly enhance antigen presentation, but both IgG1 and IgG3 present in IC's prevented antigen presentation of Der P1-(2)NIP to T cells as compared to free Der P1-(2)NIP. Results are presented as ^{3}H-thymidine incorporation on day 5 (mean + sd of triplicate wells).

Figure 3: Inhibition of antigen presentation by IgG-Der P1-(3)NIP complexes:

In the absence of IgG3, a good T cell stimulation is found when IgE-Der P1-(3)NIP complexes are used. Titration of preformed IgG3-Der P1-(3)NIP complexes in the same (1:1) ratio leads to a dose-dependent inhibition of IgE-mediated antigen presentation. In order to have this effect the IgG3 complexes need to be present in the culture medium at the time of T cells stimulation. Preincubation or "pulsing" of the B cells with the IgG3 complexes is not successful due to the low affinity of IgG3 for CD32 on the B cells. A similar inhibition is seen with antigen presentation in the absence of IgE and also with IgG3-BSA-(3)NIP complexes.

Figure 4: IgG3-mediated antigen presentation by fresh human monocytes:

Human monocytes are pulsed with preformed IgG3-Der P1-(3)NIP complexes (ratio 1:1) for 1 hour on ice in the absence or presence of non-relevant aggregated human IgG. The pulsed monocytes are subsequently washed and mixed with HLA-DP matched T cell clones specific for Der P1, and after 5 days proliferation is measured as ^{3}H-thymidine incorporation. IgG3 complexes presented by human monocytes induce an allergen-specific T cell response which can be blocked by aggregated IgG, indicating specificity of the IgG3 interaction with the monocytes. Data are shown as mean $\pm$ sd of triplicate wells.

Figure 5: DPT-specific T cell proliferation; influence of aCD32 or aggregated human IgG on antigen presentation:

Human EBV B cells are pulsed with (closed bars) or without (open bars) 250 µg/ml of DPT overnight. After irradiation the B cells are mixed with T cells from a Der P1 - specific T cell clone and the cells are allowed to proliferate for 5 days. In the presence of different concentrations of aCD32 antibodies cross-linked by GaM a dose-dependent inhibition of proliferation is seen. The same effect is observed when aCD32 or non-specific aggregated human IgG is coated to the culture wells.

Figure 6: Examples of recombinant fusion proteins between aCD32 scFv (Fab) fragment(s) derived from a phage display library:

The Fab fragments can be obtained from a phage display library such as described by De Kruif et al., Proc. Natl. Acad. Sci. USA 92 (1995) 3938-3942. However, any molecule (or part of a molecule) efficiently interacting with human CD32 can replace the aCD32 Fab fragment. Allergen may be any protein or combination of proteins that cause(s) allergy. In addition the allergen(s) may be replaced by fragments of the allergen(s) which contain T cell epitopes. The recombinant protein can be produced in any expression system available, independent of glycosylation and other post-translatory modifications. Such fusion proteins may also be used for diseases which are characterized by (over)production of unwanted Ig molecules such as in rheumatoid arthritis, graft-versus-host disease or any other disease in which autoantibodies play a role. In these cases allergens can be replaced by a simple non-defined linker to combine the two aCD32 moieties, however the best results will be obtained when allergen is replaced by the "autoantigen" that causes the disease.

Figure 7: Determination of Der P1 in Superose-12 purified Der P1--Mc.a-CD32 conjugate fraction pools by Sandwich-ELISA

Figure 8: Determination of Mc.a-CD32 in Superose-12 purified Der P1--Mc.a-CD32 conjugate fraction pools by Sandwich-ELISA

Figure 9: Detection of Der P1—Mc.a-CD32 conjugate in Superose-12 purified fraction pools by Sandwich-ELISA:

Coating: Mc.a-Der P1.

Figure 10: Detection of Der P1--Mc.a-CD32 conjugate in Superose-12 purified fraction pools by Sandwich-ELISA:

Coating: Pc.a-mouse IgG2b.

Figure 11: Silver staining of fractions from Pool A, B and C:

Lane 1: 350 ng Der P1
Lane 2: 50 ng Mc.a-CD32
Lane 3: 500 ng high molecular weight markers
Lane 4: 500 ng starting material for Superose-12
Lane 5: 100 ng Superose-12 Pool A
Lane 6: 100 ng Superose-12 Pool B
Lane 7: 200 ng Superose-12 Pool C
Lane 8: 250 ng high molecular weight markers

High molecular weight markers:

669 kD : thyroglobulin
440 kD : ferritin
232 kD : catalase
140 kD : lactate dehydrogenase
67 kD : bovine serum albumin

Figure 12: Silver staining of purified fusion protein from Pool C:

Lane 1: 250 ng high molecular weight markers
Lane 2: 45 ng FPLC Mono-S purified material.

Figures 13a and 13b: Ag-specific T cell proliferation:

Fig. 13a: APC - Mo 250495 and CFB4:2
Fig. 13b: Influence of monomeric purified CD32/Der P1

Chemically linked aCD32 (Medarex clone IV.3) with purified Der P1 was used to stimulate Der P1 - specific T cell clone CFTS4:3.1 in different concentrations using standard LST [Van Reijsen, F. C. et al. (1992) J. Allergy Clin. Immunol. 90 184]. In a first set of experiments (Fig. 13a) pool A and pool B from the chemically fused preparation were used: pool A containing a single band of 700 kD (being 2 aCD32 molecules fused with 10 Der PI molecules) and pool B consisting of two bands at 460 kD and 330 kD (being 1 aCD32 fused with 10 Der P1, and 1 aCD32 with 5 Der PI molecules, respectively).

In a second set of experiments (Fig. 13b) the purified protein (CP230595) from pool C was used, consisting of a single band of approximately 330 kD (1 aCD32 molecule fused with 5 molecules of Der P1).

Monocytes and B cells were preincubated for 1hour at room temperature with the various fractions as indicated in the Figure. As control stimulation 100 µg/ml DPT was added to T cells plus antigen-presenting cells during the complete culture

period. T cell stimulation was measured as ^{3}H-thymidine incorporation (mean ± sd of 4 wells) after 5 days as described (Van Reijsen et al., supra). B cells were able to stimulate CFTS4:3.1 with DPT but not with the pool A, pool B, or pool C, whereas monocytes were able to stimulate CFTS4:3.1 with DPT and with pool A, pool B, and pool C. This corresponds to previous findings with natural IgG allergen complexes [Bheekha Escura, R. et al., Immunology (1995) 86 343]. In addition this indicates that for antigen stimulation by monocytes crosslinking of more than 2 CD32 molecules is not necessary, since all fractions were equally effective at stimulating the T cells.

Figure 14: Inhibition of IgE and, respectively, IgG1 synthesis:

Purified human tonsillar B cells were stimulated with aCD40 and IL-4 as described in Armerding, D. et al. Immunobiology 188 (1993) 259-273 in the presence and absence of commercially available aCD32 antibodies added in the indicated concentrations. After 9 days the supernatants of the cultures were tested for IgE and IgG1 content as described (Armerding, D. et al., supra). Results are shown as antibody production of 3 pooled fractions of 9 replicates (mean ± sd). All aCD32 antibodies inhibited IgE as well as IgG1 production in a dose-dependent way. Inhibition of IgM and IgA was comparable.

Figure 15: Inhibition of IgE synthesis:

Purified human tonsillar B cells were stimulated with aCD40 and IL-4 as described in Armerding, D. et al., supra in the presence and absence of a commercially available aCD32 antibody (Medarex IV.3) or an Fab fragment thereof, added in the indicated concentrations. After 9 days the supernatants of the cultures were tested for IgE and IgG1 content as described (Armerding, D. et al., supra). Results are shown as antibody production of 3 pooled fractions of 9 replicates (mean ± sd). Both complete and Fab fragments from the Medarex clone IV.3 were able to inhibit IgE synthesis (IgG1, IgM and IgA).

Figure 16: DPT specific IgE induction: influence of aCD32:

Human tonsilar B cells are pulsed with (closed bars) or without (open bars) 250 µg/ml of DPT overnight. After irradiation the B cells are mixed with T cells from a Der P1 - specific T cell clone (CFTS4:3.1) and the cells are allowed to proliferate and produce antibodies for 9 days. After 9 days the supernatants of the cultures were tested for IgE and IgG1 content as described in Armerding, D. et al., supra. Results are shown as antibody production of 3 pooled fractions of 9 replicates (mean ± sd). In the presence of different concentrations of aCD32 antibodies a dose-dependent inhibition of IgE production is seen. This indicates that even in cognate B cell T cell interaction, aCD32 antibodies block antibody synthesis.

Sequence listing

(1) GENERAL INFORMATION:

(i) APPLICANT:

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(F) POSTAL CODE (ZIP): A-1230

(ii) TITLE OF INVENTION: FUSION PROTEINS

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0,
Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: WO PCT/EP96/ ....

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: GB 9516760.7

(B) FILING DATE: 16-AUG-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 43 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Dermatophagoides pteronyssinus

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(D) TOPOLOGY: unknown

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(vi) ORIGINAL SOURCE:

(A) ORGANISM: Dermatophagoides pteronyssinus

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Claims:

1. Fusion protein comprising
   a) one or more antigens and
   b) one or more moieties interacting with human Fcγ receptor II (FcγRII) (CD32).

2. Fusion protein according to claim 1 in which the antigens are allergens in atopic dermatitis, allergic asthma, allergic rhinitis or allergic conjunctivitis.

3. Fusion protein according to claim 1 which is produced from one or more T cell epitope - containing DNA stretches, rather than from the genes for the complete antigens.

4. Fusion protein according to any one of claims claims 1 to 3 in which the moieties interacting with FcγRII are human or humanized aCD32 antibodies, or parts of these antibodies which still specifically recognize and bind to FcγRII (CD32) antigen.

5. Fusion protein according to any one of claims 1 to 3 in which the moieties interacting with FcγRII is/are human or humanized IgG antibodies, or parts of these antibodies which still interact with FcγRII (CD32) antigen.

6. Fusion protein according to any one of claims 1 to 3 in which the moieties interacting with FcγRII is/are manipulated human or humanized aCD32 or IgG antibodies, or parts thereof, which recognize FcγRII (CD32) with higher affinity than the native aCD32 or IgG antibodies.

7. Pharmaceutical composition comprising a fusion protein according to claim 1 together with at least one pharmaceutically acceptable carrier or diluent.

8. Process for producing a fusion protein according to claim 1 which comprises the use of recombinant gene techniques or chemical cross-linking.

9. Use of a fusion protein according to claim 1 in the prevention and/or treatment of allergies (including food allergies).

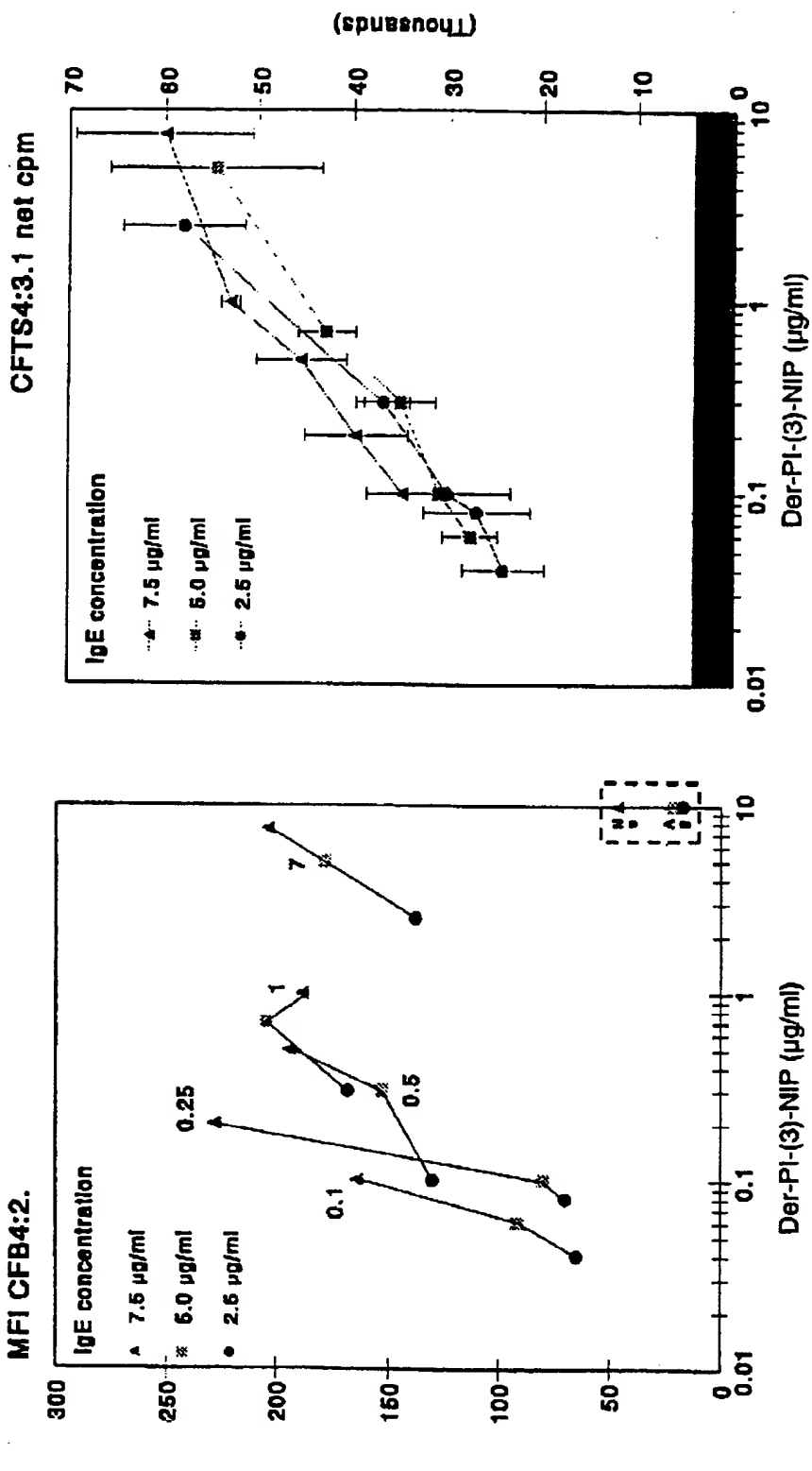
10. Use of a fusion protein according to claim 1 for the manufacture of a medicament for the prevention and/or treatment of allergies (including food allergies).

11. Fusion protein according to claim 1 for use as a pharmaceutical.

12. Method of treating allergies which comprises administering to a subject in need of such treatment a prophylactically or therapeutically effective amount of fusion protein according to claim 1 together with at least one conventional pharmaceutically acceptable carrier or diluent.

13. Process for the preparation of a medicament against allergies which comprises mixing a fusion protein according to claim 1 together with a pharmaceutically acceptable carrier or diluent.

Figure 1: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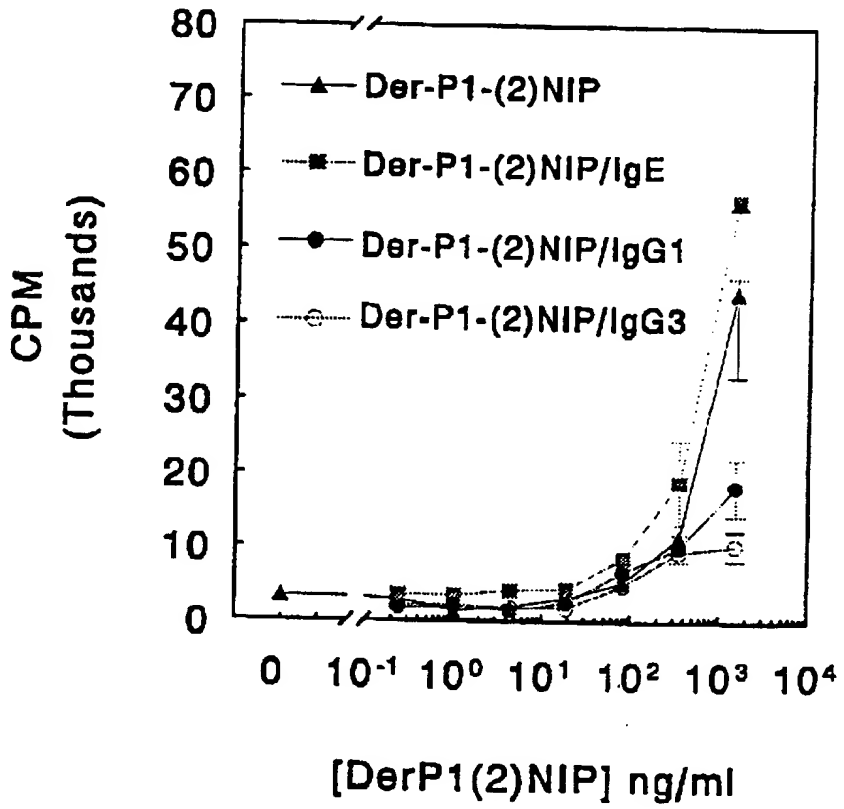
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(Thousands)
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Der-P1-(2)NIP/IgE
Der-P1-(2)NIP/IgG1
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103
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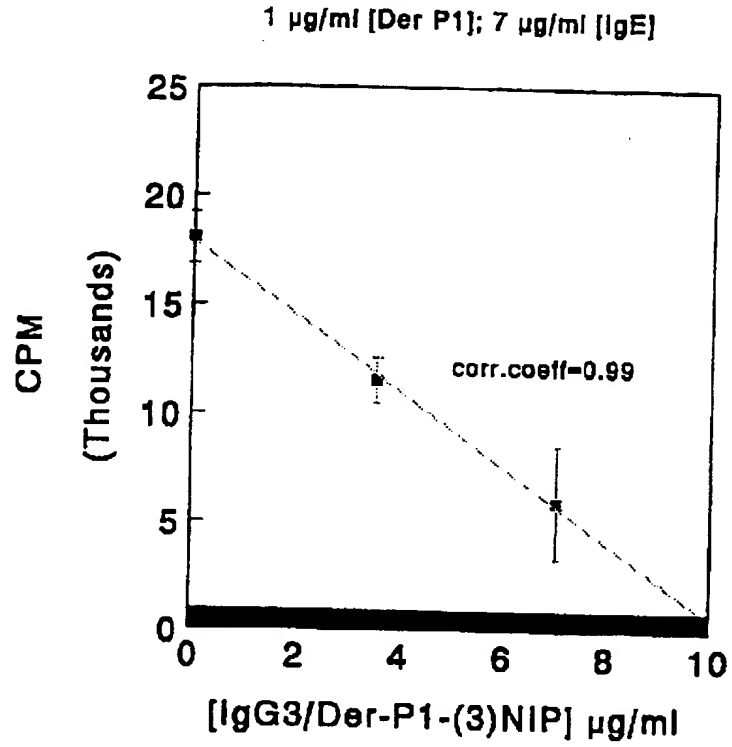
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CPM
(Thousands)
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15
10
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0
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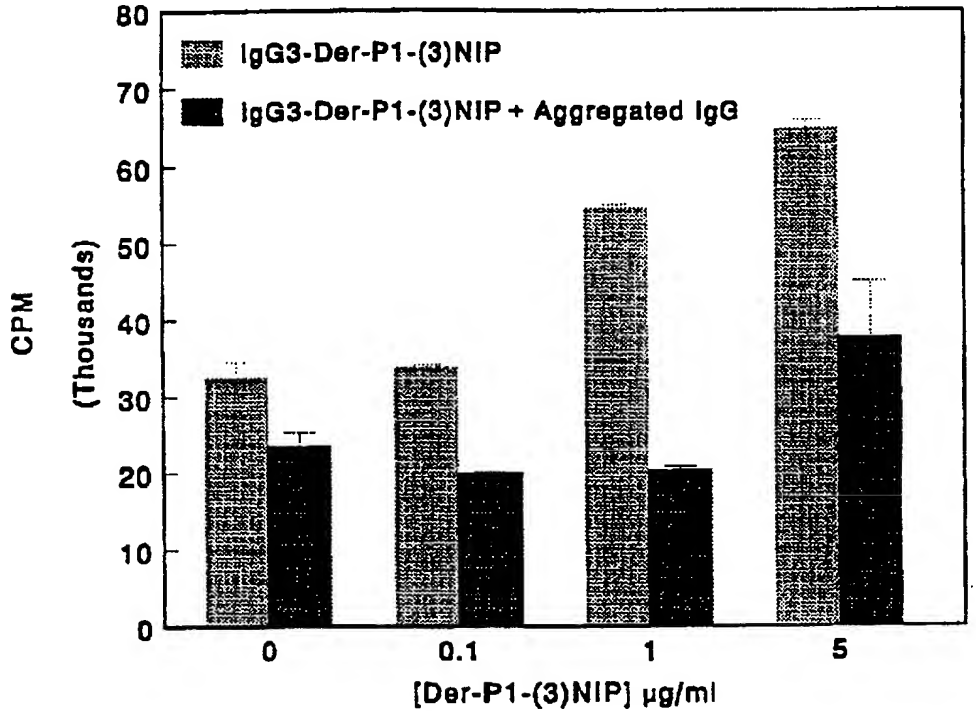
4
6
8
10
[IgG3/Der-P1-(3)NIP] µg/ml

Figure 4: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IgG3-Der-P1-(3)NIP + Aggregated IgG
CPM
(Thousands)
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20
10
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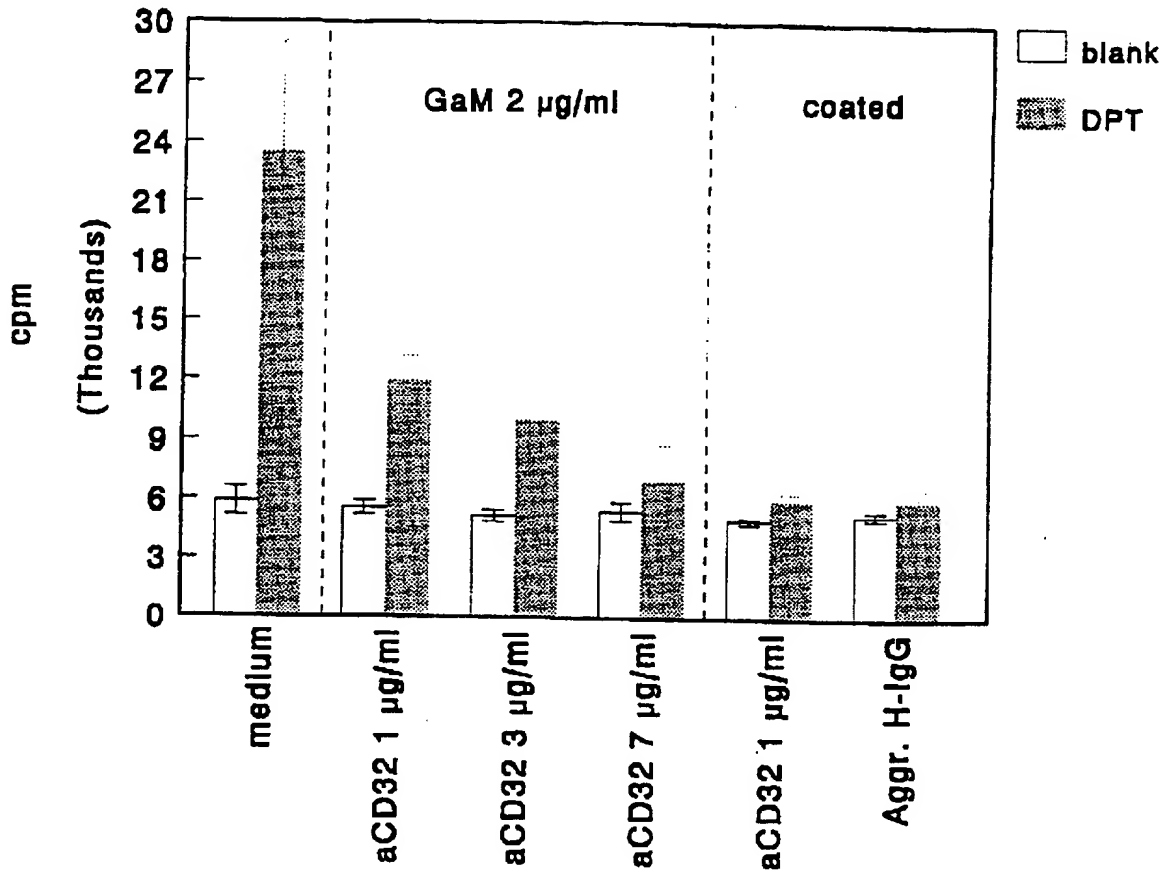
0
0.1
1
5
[Der-P1-(3)NIP] µg/ml

Figure 5: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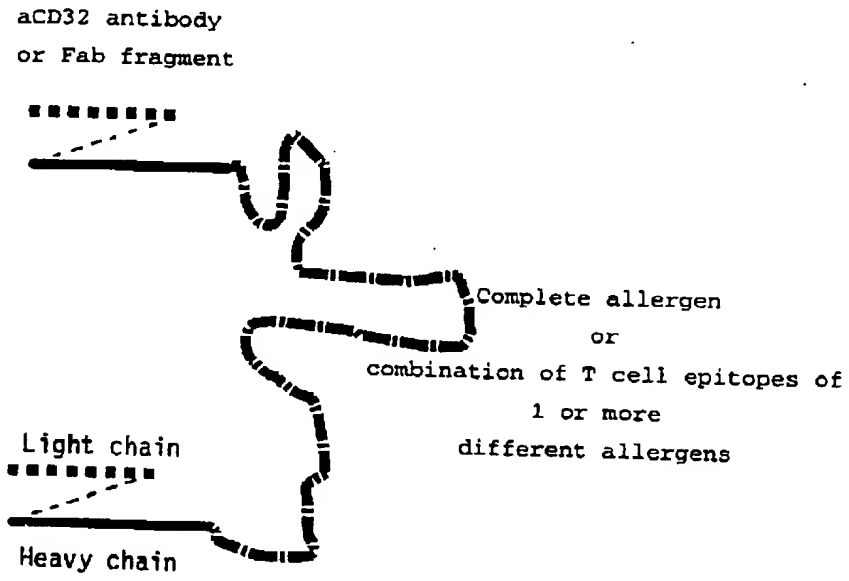
0
cpm
(Thousands)
GaM 2 µg/ml
coated
blank
DPT
medium
aCD32 1 µg/ml
aCD32 3 µg/ml
aCD32 7 µg/ml
aCD32 1 µg/ml
Aggr. H-IgG

Figure 6:

Fusion protein consisting of aCD32 Fab-allergen-aCD32 Fab



Fusion protein consisting of aCD32 Fab-allergen or allergen-aCD32 Fab

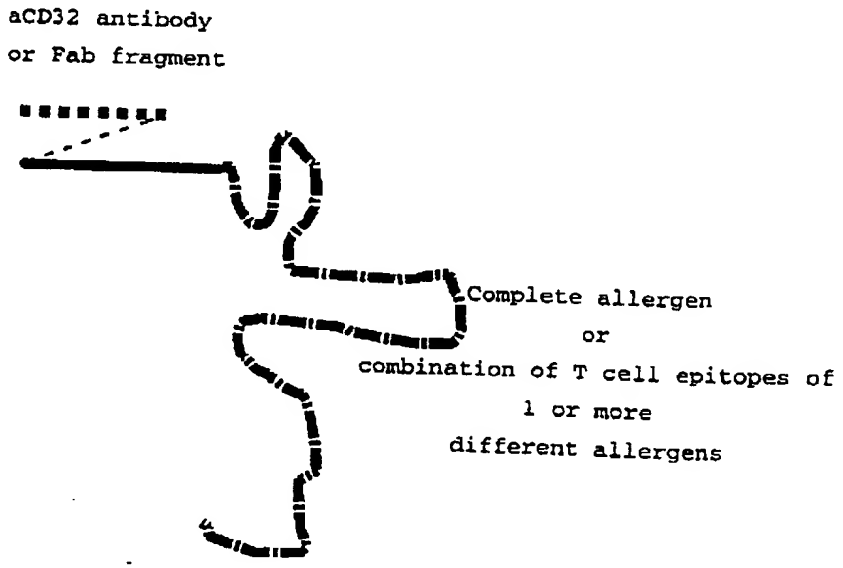


Figure 7:

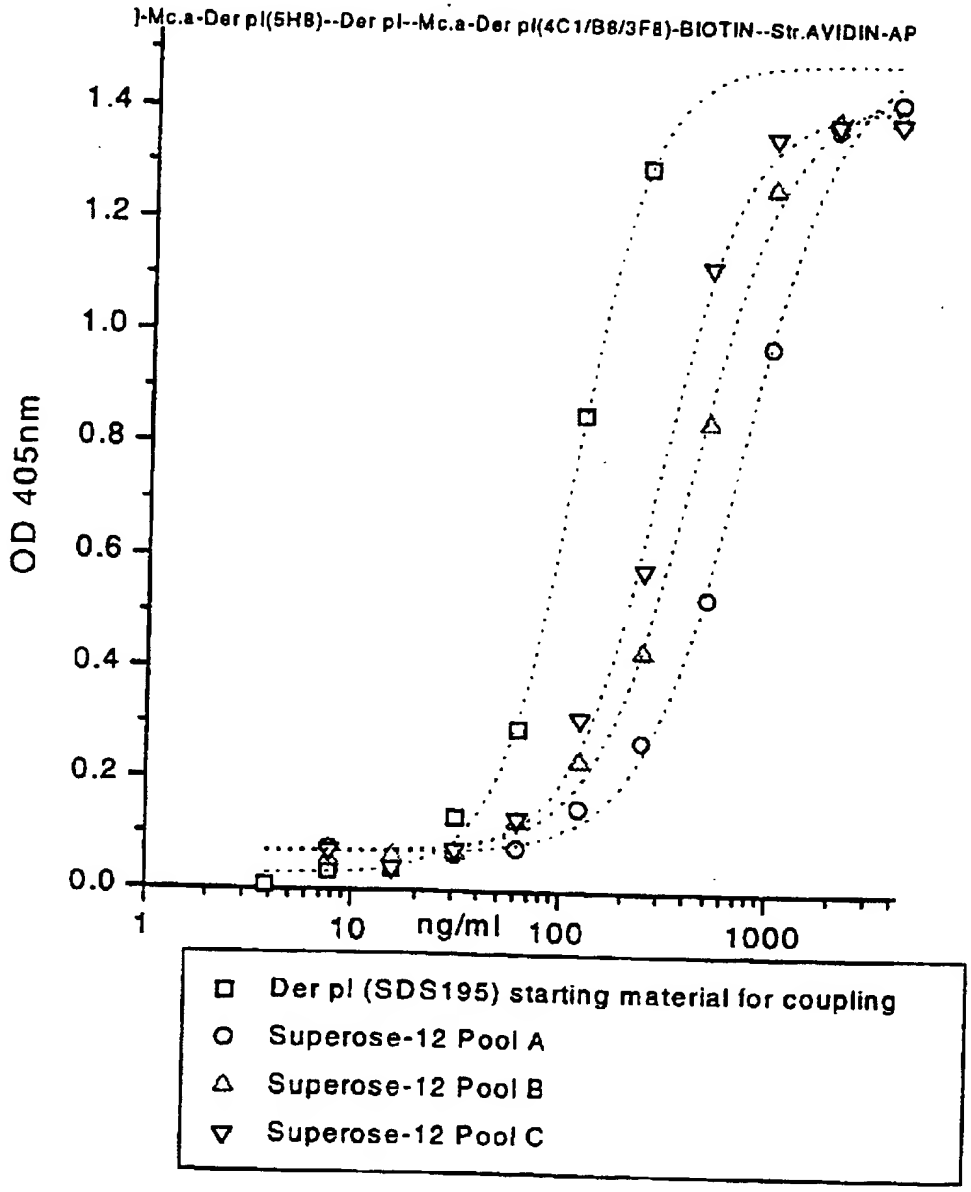

]-Mc.a-Der pl(5H8)--Der pl--Mc.a-Der pl(4C1/B8/3F8)-BIOTIN--Str.AVIDIN-AP
OD 405nm
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
1
10
ng/ml
100
1000
Der pl (SDS195) starting material for coupling
Superose-12 Pool A
Superose-12 Pool B
Superose-12 Pool C

Figure 8:

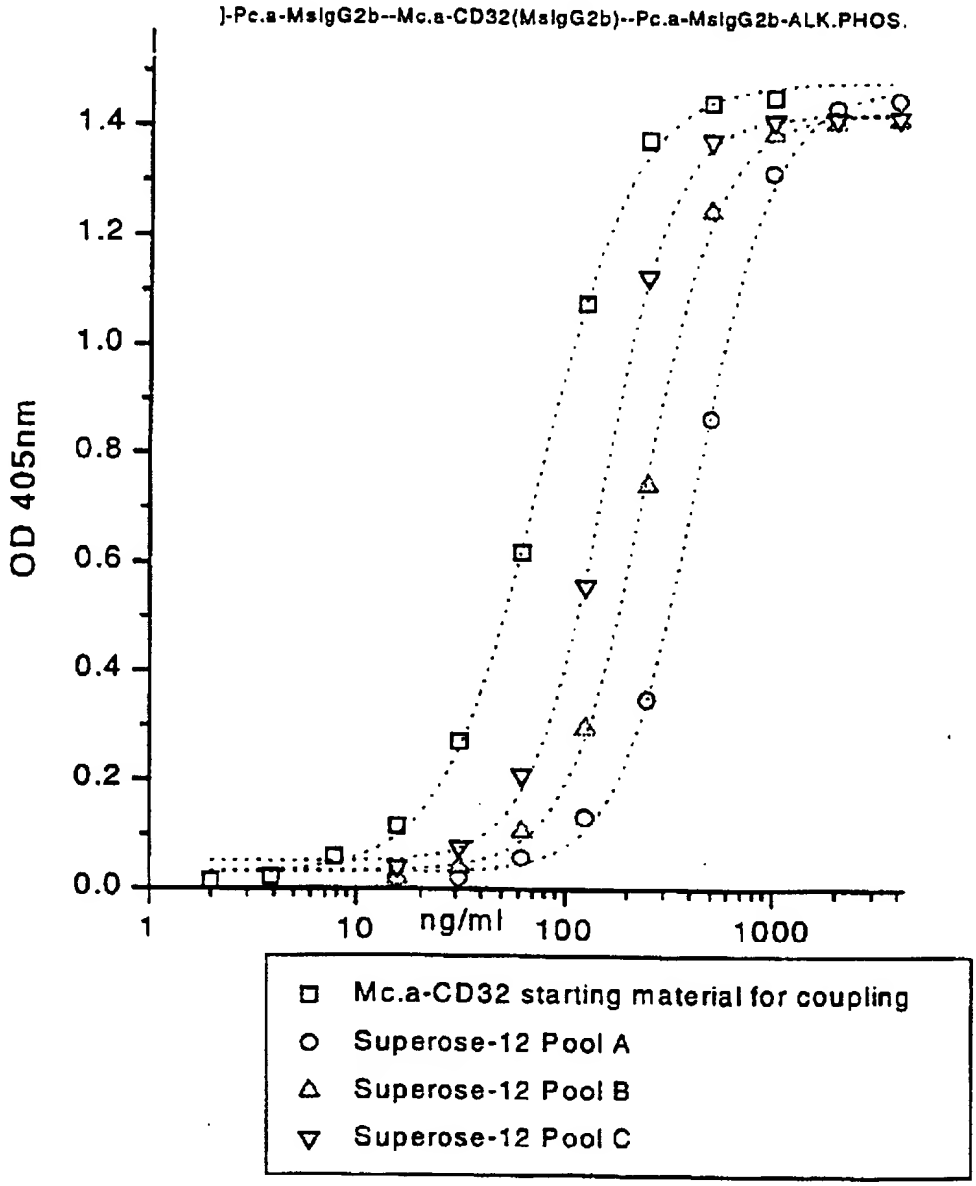

}-Pc.a-MsIgG2b--Mc.a-CD32(MsIgG2b)--Pc.a-MsIgG2b-ALK.PHOS.
OD 405nm
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1
10
ng/ml
100
1000
Mc.a-CD32 starting material for coupling
Superose-12 Pool A
Superose-12 Pool B
Superose-12 Pool C

Figure 9:

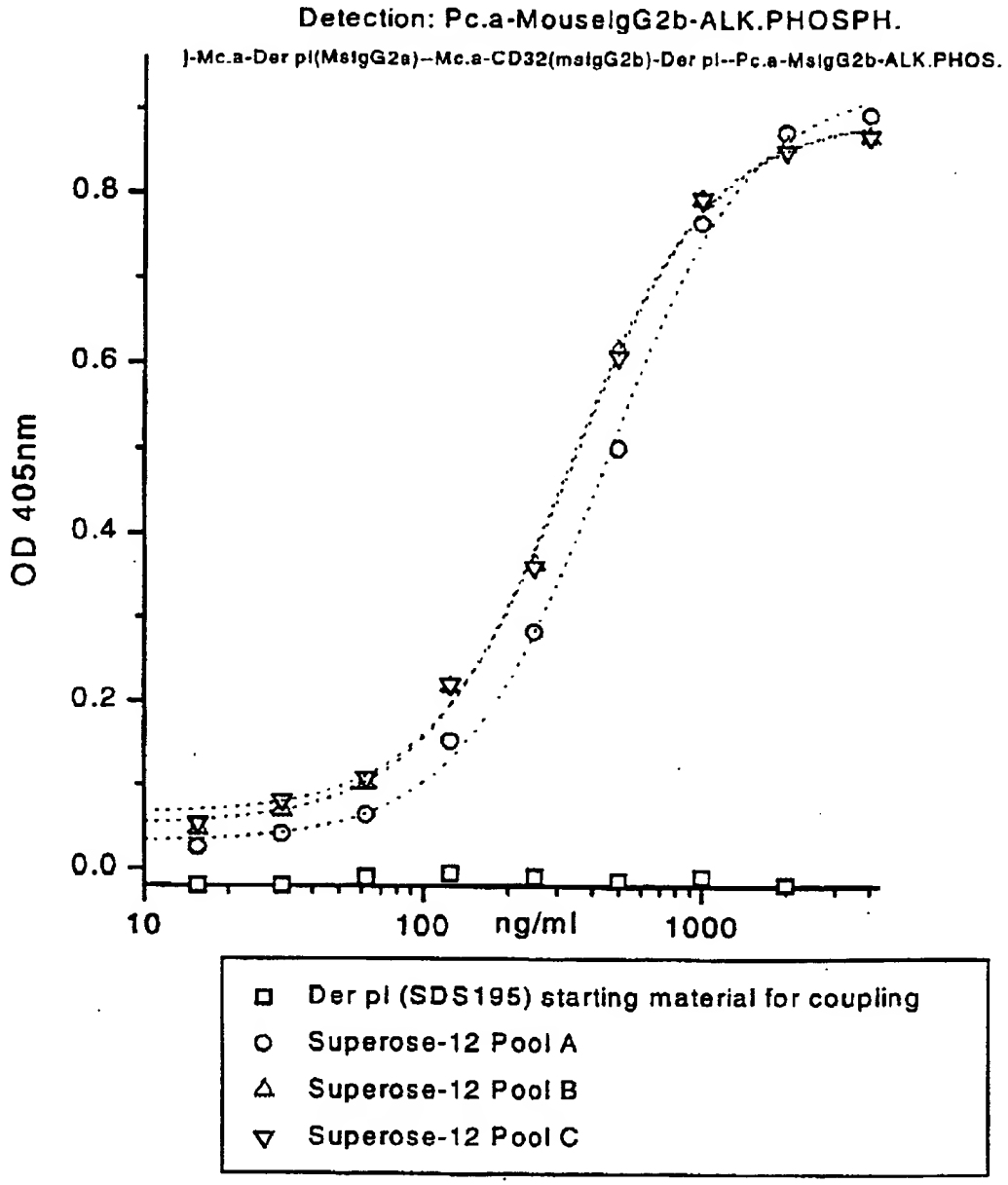

Detection: Pc.a-MouseIgG2b-ALK.PHOSPH.
]-Mc.a-Der pl(MsIgG2a)--Mc.a-CD32(msIgG2b)-Der pl--Pc.a-MsIgG2b-ALK.PHOS.
OD 405nm
0.0
0.2
0.4
0.6
0.8
10
100
ng/ml
1000
Der pl (SDS195) starting material for coupling
Superose-12 Pool A
Superose-12 Pool B
Superose-12 Pool C

Figure 10:

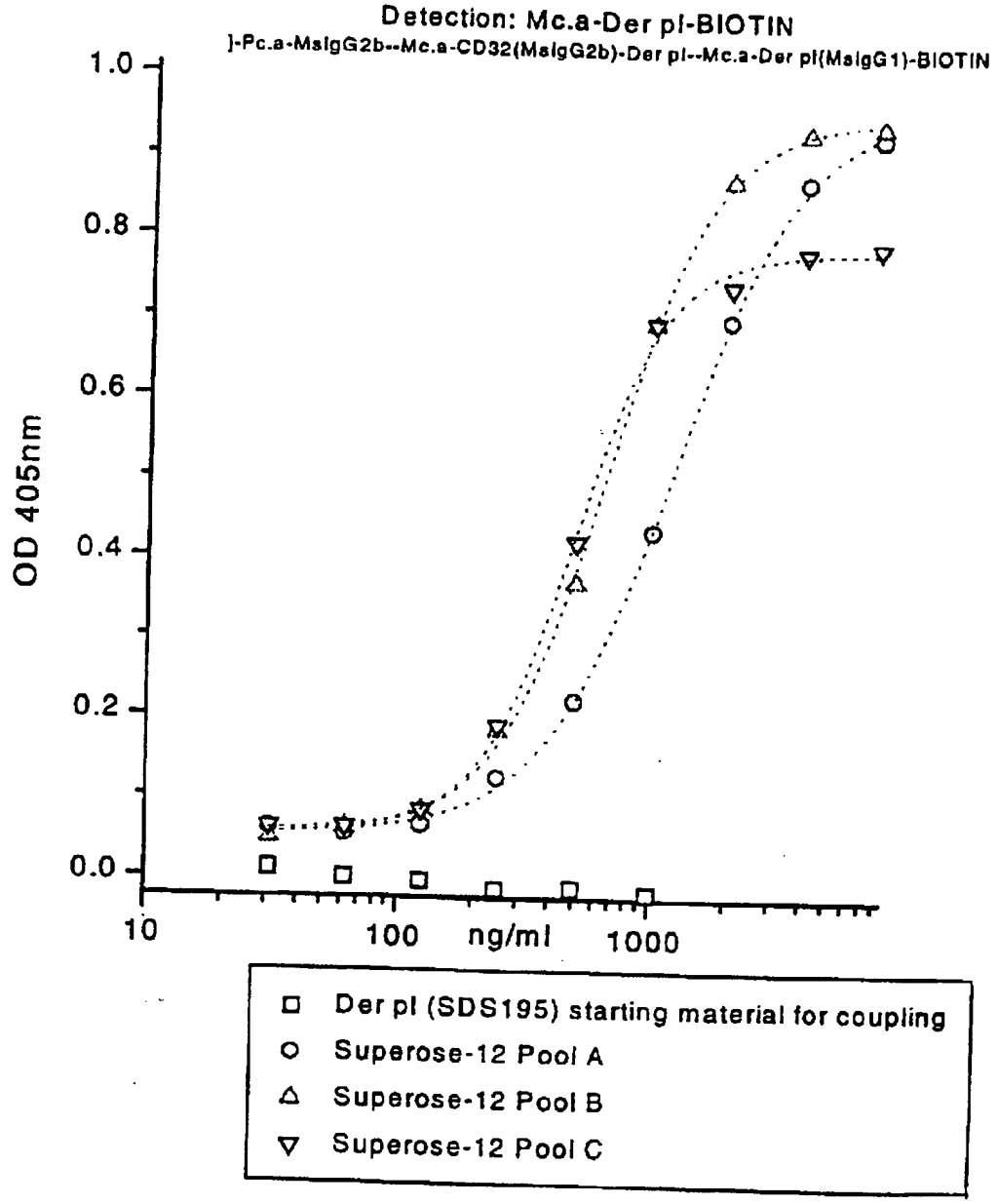

Detection: Mc.a-Der pI-BIOTIN
]-Pc.a-MsIgG2b--Mc.a-CD32(MsIgG2b)-Der pI--Mc.a-Der pI(MsIgG1)-BIOTIN
OD 405nm
1.0
0.8
0.6
0.4
0.2
0.0
10
100
ng/ml
1000
Der pI (SDS195) starting material for coupling
Superose-12 Pool A
Superose-12 Pool B
Superose-12 Pool C

Figure: 11:

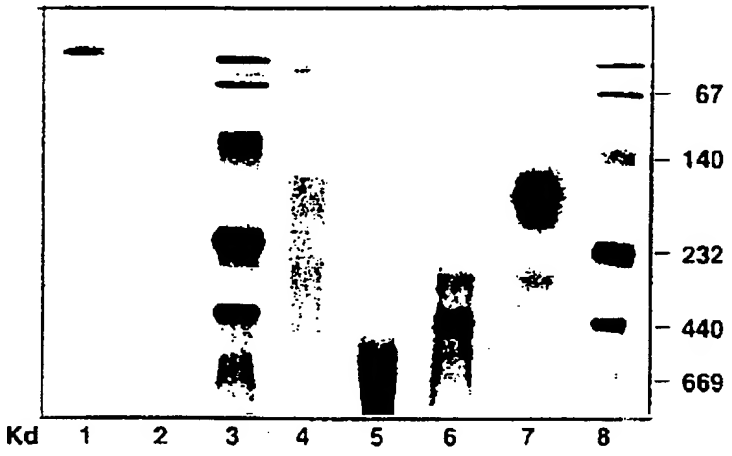


Figure 12:

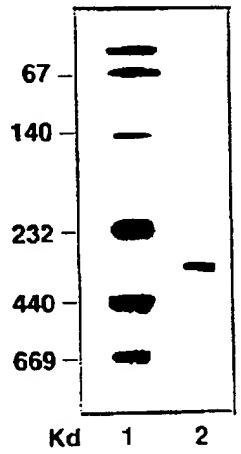


Figure 13a:

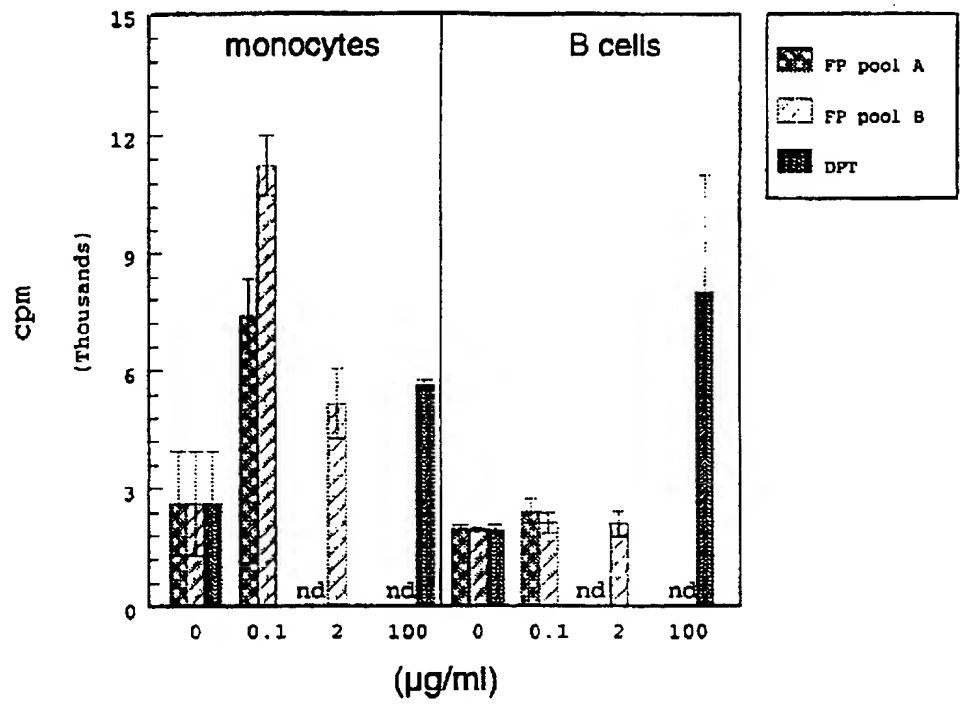


Figure 13b:

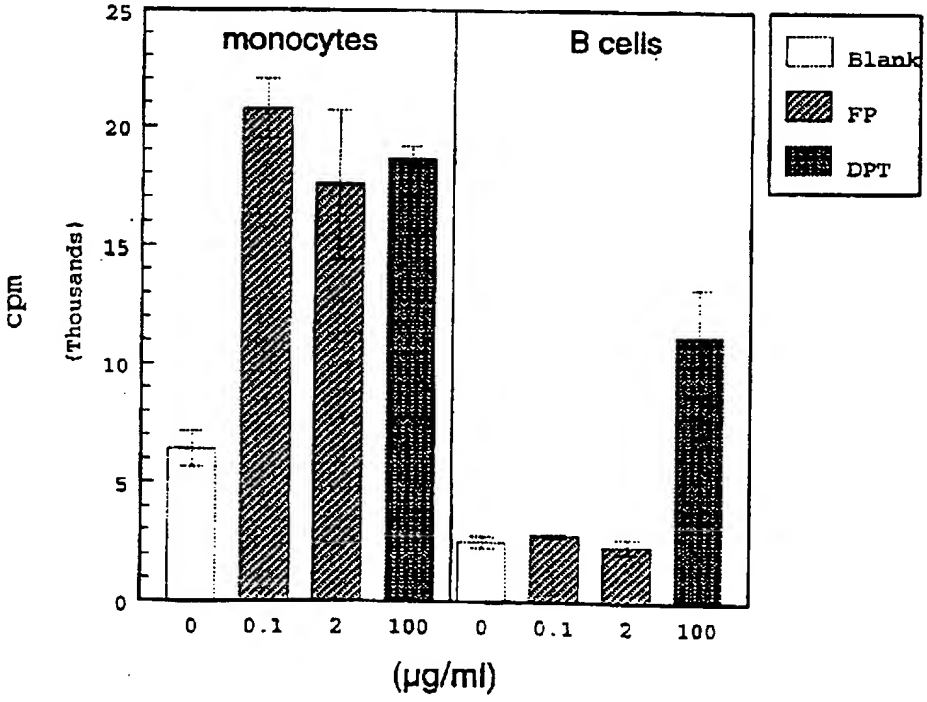
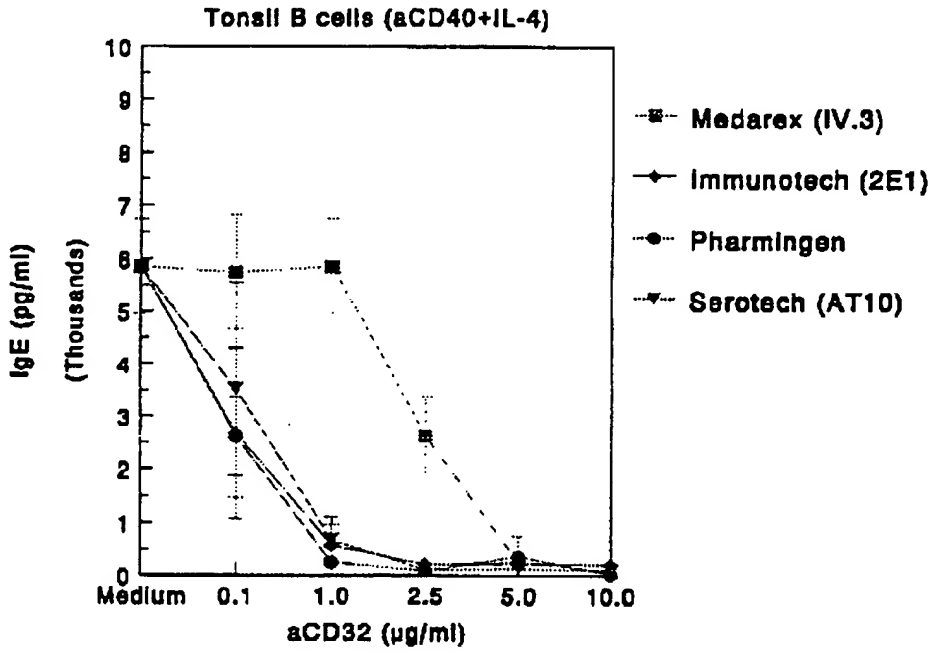


Figure 14:


Tonsil B cells (aCD40+IL-4)
IgE (pg/ml)
(Thousands)
10
9
8
7
6
5
4
3
2
1
0
Medium
0.1
1.0
2.5
5.0
10.0
aCD32 (µg/ml)
Medarex (IV.3)
Immunotech (2E1)
Pharmingen
Serotech (AT10)

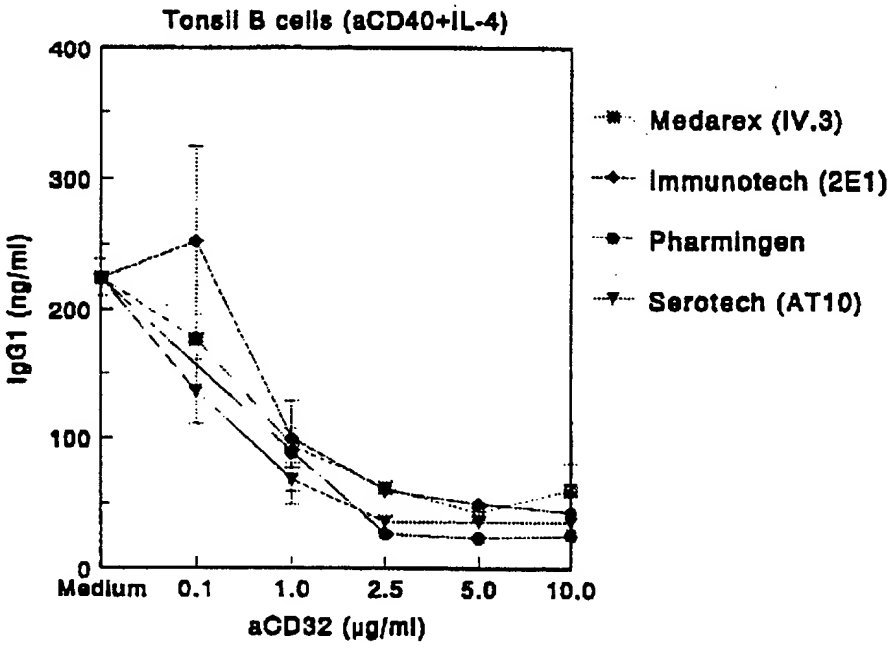

Tonsil B cells (aCD40+IL-4)
IgG1 (ng/ml)
400
300
200
100
0
Medium
0.1
1.0
2.5
5.0
10.0
aCD32 (µg/ml)
Medarex (IV.3)
Immunotech (2E1)
Pharmingen
Serotech (AT10)

Figure 15:

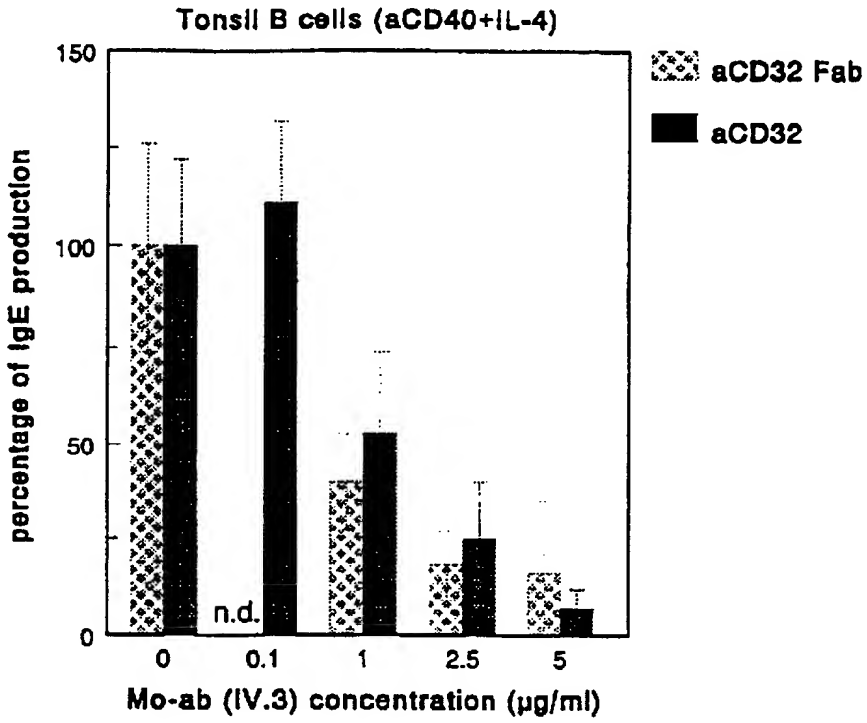
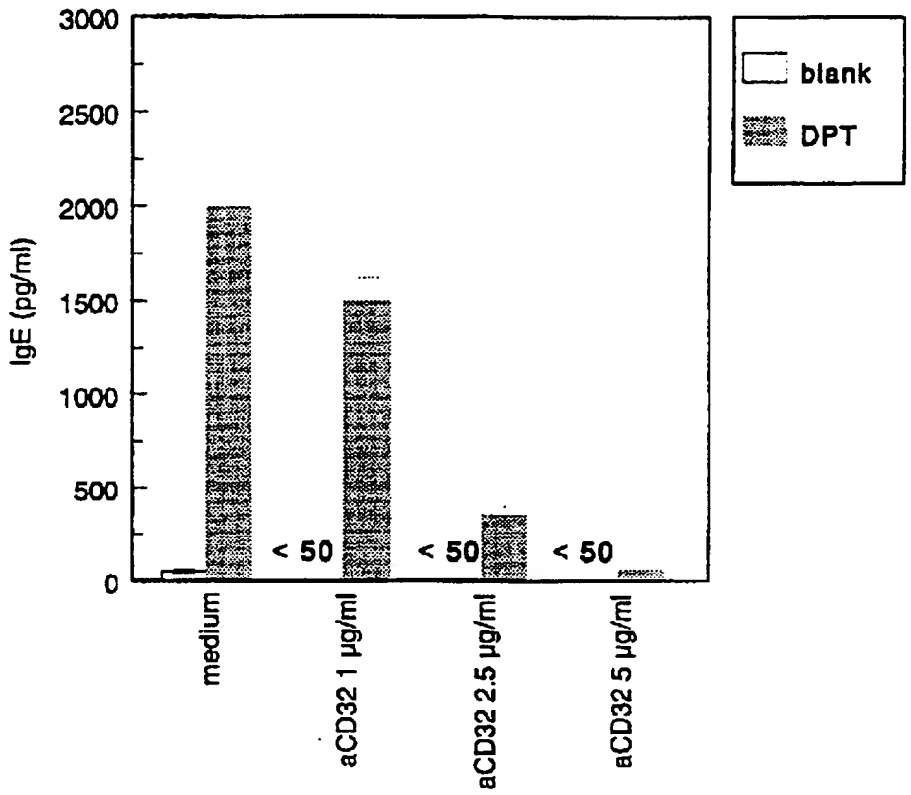

Tonsil B cells (aCD40+IL-4)
percentage of IgE production
150
100
50
0
n.d.
0
0.1
1
2.5
5
Mo-ab (IV.3) concentration (µg/ml)
aCD32 Fab
aCD32

Figure 16:


3000
2500
2000
1500
1000
500
0
IgE (pg/ml)
blank
DPT
< 50
< 50
< 50
medium
aCD32 1 µg/ml
aCD32 2.5 µg/ml
aCD32 5 µg/ml

INTERNATIONAL SEARCH REPORT

National Application No
PCT/EP 96/03616

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/62 C07K19/00 C07K16/28 C07K14/435 A61K39/35 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 24281 (IMMULOGIC PHARMA CORP) 27 October 1994 see the whole document ---	2
P,X	WO,A,95 32734 (INNOGENETICS NV ;BOER MARK DE (NL); BARCY SERGE (BE)) 7 December 1995 page 14 line 10-30; page 15, line 2 ff.; claims 4-6 ---	1-6,8,10
P,X	IMMUNOLOGY, vol. 86, November 1995, pages 343-350, XP002020270 BHEEKHA ESCURA R. ET AL.,: "Regulation and targeting of T-cell immune responses by IgE and IgG antibodies" Summary and page 349, paragraph 3 - page 350 end of document. -----	1-3,5,6, 8

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 December 1996	17. 12. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Müller, F

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/EP 96/03616

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 9 and 12 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

.national Application No
PCT/EP 96/03616

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9424281	27-10-94	ZA-A-	9302677	15-11-93
		AU-A-	4102693	08-11-94
		EP-A-	0694067	31-01-96
		FI-A-	954895	13-10-95
		FI-A-	963331	27-08-96
		NO-A-	954095	13-12-95
WO-A-9532734	07-12-95	AU-A-	2670995	21-12-95